Laboratory Guidelines for the Detection and Diagnosis of Monkeypox Virus Infection

23 May 2022

This document is based on World Health Organization interim guidance on Laboratory testing for monkeypox virus, 23 May 2022, and is intended to provide guidance to National Reference Laboratories on monkeypox virus laboratory detection.

Monkeypoxvirus (MPXV) is a double-stranded DNA virus, a member of the *Orthopoxvirus* genus within the *Poxviridae* family. Poxviruses cause disease in humans and many other animals; infection typically results in the formation of lesions, skin nodules or disseminated rash. All orthopoxviruses (OPXV) are antigenically related and other species pathogenic to humans include cowpox virus and variola virus (causing smallpox, which has been eradicated). Vaccinia virus is also an OPXV that has been used as an attenuated vaccine and was a key tool for the eradication of smallpox, achieved in 1980.

MPXV is named due to its initial detection in monkeys and can primarily be found in rodents; however, the reservoir is undetermined. There are two known clades of MPXV, one endemic in Western Africa and one in the Congo Basin region.

After an incubation period ranging from 6 to 16 days, the typical presentation of monkeypox initiates with a short febrile prodromal period followed by progressive development of a classic rash with indurated and umbilicated (centrally depressed) lesions, starting on the head or face and progressing to the limbs and trunk. Lesions progress all at the same stage from macules, to papules, to vesicles, to pustules and eventually to crusts which dry up and fall off after two to four weeks. There are often enanthems (sores or ulcers) in the mouth and lesions can affect the eyes and/or genital area.

Because of range of conditions that cause skin rashes and because clinical presentation may more often be atypical in this outbreak, it can be challenging to differentiate monkeypox solely based on the clinical presentation. Therefore, decision to test should be based on both clinical and epidemiological factors, linked to an assessment of the likelihood of infection.

Given the current multiple detection of MXPV world-wide, any individual meeting the definition for a suspected case should be offered testing. In this sense, the Pan American Health Organization / World Health Organization (PAHO/WHO) recommends to Member States to ensure the timely identification of suspect cases, the timely collection of samples and the implementation of molecular detection protocols at the National Public health Laboratories according to the existing capacity. When necessary, shipping of samples to Regional or Global Reference laboratories might be considered. Contact PAHO Regional Office for further advice and procedures.

SAMPLE COLLECTION AND MANAGEMENT

Safety procedures

Use of adequate standard operating procedures (SOPs) must be ensured, and laboratory personnel must be trained for appropriate use of personal protective equipment (PPE) including disposable antifluid gown, latex gloves, goggles or full-face cover, lab hat, and shoe covers, and for the elimination of used PPE. Additionally, staff should be appropriately trained for specimen collection, storage, packaging, and transport.

Biological risk management

Measures should be taken to minimize the risk of laboratory transmission based on a **risk assessment at institutional level** when testing routine clinical specimens from confirmed or suspected monkeypox patients. These may include limiting the number of staff testing specimens only to staff with proven competency, wearing appropriate PPE, using rigorously applied standard precautions, using effective disinfectants (which include quaternary ammonium compounds and 0.5% (or 200ppm) bleach (0.5%), and avoiding any procedures that could generate aerosols.

Rigorous adherence to infection prevention and control guidelines must be ensured during specimen collection and handling.

It is recommended that all manipulations of specimens originating from suspected, probable or confirmed cases of monkeypox in the laboratory be conducted according to a risk-based approach. Each laboratory should conduct an institutional risk assessment.

When manipulating biological specimens, core biosafety requirements, similar to those previously referred to as biosafety level 2, must be met and heightened control measures should be applied based on local risk assessment.

MPXV may be contracted at the lab during the specimen processing stage from contaminated material or inadequate lab practices. Therefore, heightened biosafety measures are recommended in addition to the core requirements, including the following for the purpose of clinical testing without virus propagation:

- Specimens from patients with suspected MPXV infection must be handled in a reviewed (according to the PAHO laboratory maintenance manual), or certified Class II biosafety cabinet, prior to sample inactivation. Properly inactivated specimens do not require a biosafety cabinet.
- Laboratory personnel should wear appropriate PPE, especially for handling specimens before inactivation.
- Where use of a centrifuge is required for a procedure, safety cups or sealed rotors should be used.

Additional control measures should be considered for specific procedures, including aerosol-generating procedures, according to the local risk assessment. For more information on core biosafety requirements and heightened control measures, please see the fourth edition of the WHO Biosafety Manual.

Types of samples

The recommended specimen type for laboratory confirmation of monkeypox is skin lesion material, including:

- Swabs of lesion surface and/or exudate,
- Roofs from more than one lesion, or
- Lesion crusts.

Lesions swabs, crusts and vesicular fluids should not be mixed in the same tube.

Swab the lesion vigorously using Dacron or polyester flocked swabs, to ensure adequate viral DNA is collected. Both dry swabs and swabs placed in viral transport media (VTM) can be used. Two lesions of the same type should be collected in one single tube, preferably from different locations on the body and which differ in appearance.

In addition to a lesion specimen, the collection of an oropharyngeal swab is encouraged. However, data on the accuracy of this specimen type for diagnosis is limited for monkeypox, therefore a negative throat swab specimen should be interpreted with caution.

Because the current outbreak is still under investigation, collection of additional specimen types for research purposes can be considered if allowed by the appropriate ethical review board, and there is sufficient laboratory and medical expertise for their safe collection, handling, and storage. These may include urine, semen, rectal and/or genital swab on indication based on clinical presentation including location of lesions.

Samples storage

Samples should be refrigerated (2 to 8°C) or frozen (-20°C or lower) within one hour after collection. If transport exceeds 7 days for the sample to be tested, specimens should be stored at -20°C or lower.

Longer term specimen storage (>60 days from collection) is recommended at -70°C. Repeated freeze-thaw cycles should be avoided because they can reduce the quality of specimens.

Other sample types

Additional specimens type (not intended for routine diagnostic and do not need to be collected outside of research settings) are (1) EDTA blood that can be used to support MPXV detection of but may not contain the high level of virus found in lesion samples, as any viremia occurs early in the course of infection, usually in the prodromal period, and before skin lesions become manifest; (2) lesion biopsy during the macular stage that should be considered only if clinically indicated and only be performed by personnel with appropriate training.

SHIPMENT OF SAMPLES

Specimens should be stored refrigerated or frozen within an hour of collection and transported to the laboratory as soon as possible after collection. Correct handling and storage of specimens during transportation is essential for accurate diagnostic testing.

Transport of specimens should comply with any applicable national and/or international regulations, including the UN Model Regulations regarding the recommendations for transport of dangerous substances, and any other applicable regulations depending on the mode of transport being used.

> For international transport, specimens from suspected probable or confirmed cases of MPXV should be transported as Category A, UN2814 "infectious substance, affecting humans."

All specimens being transported should have appropriate triple packaging, labelling and documentation. Shipping requires a dangerous goods certified shipper. Please see the WHO Guidance on regulations for the transport of infectious substances 2021-2022 for information on infectious substances shipping requirements (available at: https://www.who.int/publications/i/item/9789240019720)

In case of category A shipment is not available in your country, we recommend sample inactivation and shipment as exempted. Inactivation process can be carried out at the Public Health Laboratory in a level 2 biosafety environment. See additional procedure for sample inactivation at: https://www.paho.org/hq/dmdocuments/2014/2014-cha-procedures-inactivation-ebola.pdf

For international shipping, please contact PAHO Laboratory Response Team at: ricoj@paho.org or laboratoryresponse@paho.org

LABORATORY TESTING

Testing for the presence of MPXV should be performed in appropriately equipped laboratories by staff trained in the relevant technical and safety procedures. Measures should be taken to minimize the risk of laboratory transmission based on risk assessment when testing routine clinical specimens from confirmed or suspected monkeypox patients.

VIRAL ISOLATION SHOULD NOT BE ATTEMPTED

Countries with no molecular diagnostic protocol implemented for MPXV detection should send suspected clinical samples (strictly fitting case definition) to a reference laboratory designated by PAHO. For support, please contact PAHO Laboratory Response Team (ricoj@paho.org).

Molecular methods

Confirmation of MPXV infection is based on nucleic acid amplification testing (NAAT), using real-time or conventional polymerase chain reaction (PCR), for detection of unique sequences of viral DNA. PCR can be used alone, or in combination with sequencing.

Several groups have developed validated PCR protocols for the detection of OPXV and more specifically MPXV, some of which include distinction of Congo Basin and West African clades.

Some protocols involve two steps, in which the first PCR reaction detects OPXV, but does not identify which species. This can then be followed by a second step, which can be PCR-based or utilize sequencing, to specifically detect MPXV (see below, Algorithm 1). Other protocols (recommended) are based on the initial generic detection of Monkeypox (confirming the etiology), followed by specific differentiation of clades using additional PCR assays (see below, Algorithm 2).

PCR kits detecting OPXV or specifically MPXV are under development, but no commercial validated PCR kits are currently available widely.

DNA extraction

DNA can be extracted from samples mentioned above using any standard extraction protocols or kits. In general, the sample lysis step in DNA extraction inactivates any live virus.
Thus, it is recommended that the sample lysis step is performed under a biosafety cabinet. For crust samples, DNA extraction kit for tissue samples should be used to insure appropriate sample lysis.

Molecular detection

PAHO is working to support all Member States, through the national reference laboratories to implement capacity to perform molecular testing for MPXV.

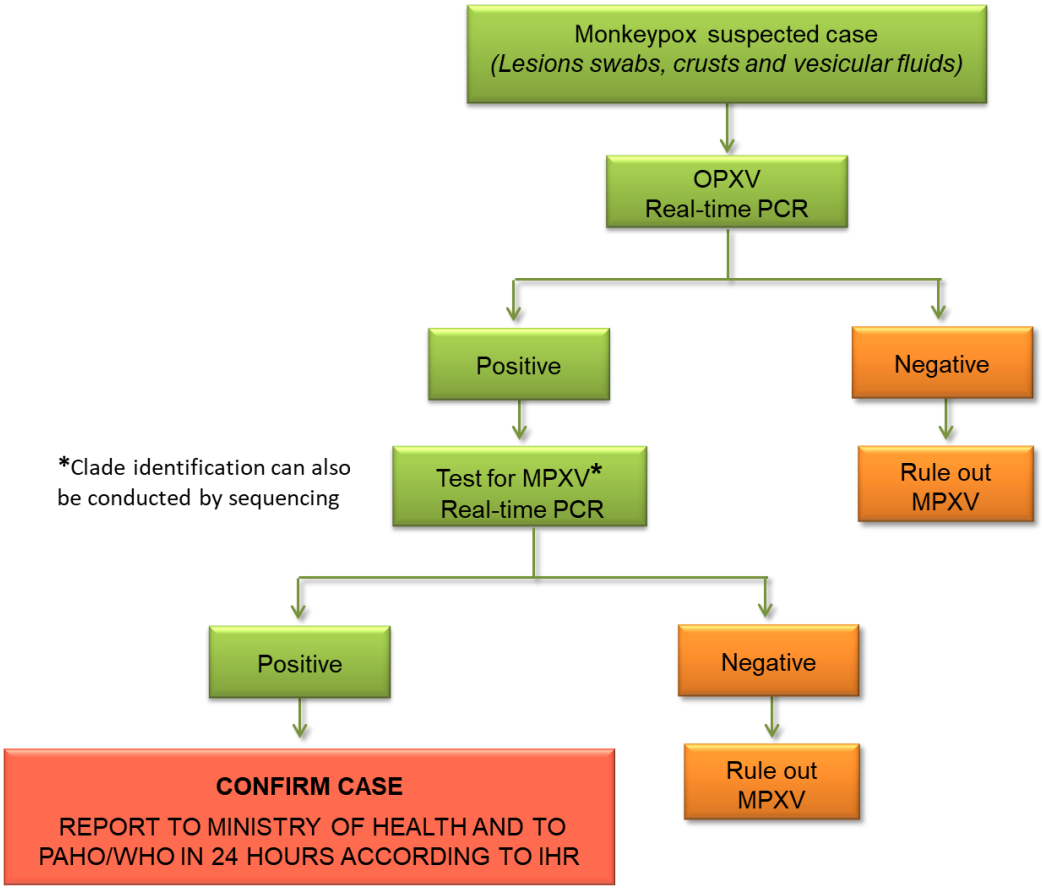
The suggested protocol (Li *et al.*, *Journal of Virological Methods.* 2010. **169**, 223–7) has been published and is available on the following link: https://doi.org/10.1016/j.jviromet.2010.07.012. A working protocol is enclosed at the end of this document (Annex 1).

This protocol is based on the initial detection of MPXV through a generic real-time PCR that detects all MPXV strains. If positive, it is followed by subsequent reactions targeting specifically the two MPXV clades: Western Africa and Congo Basin.

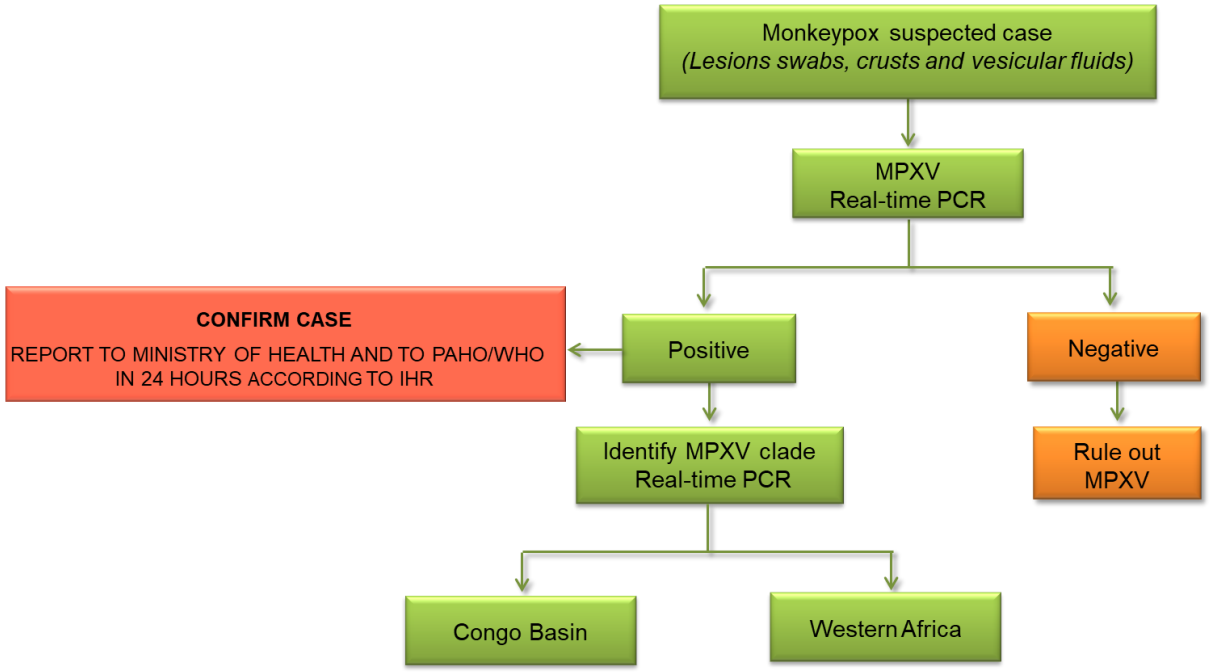
Specific reagents (primers, probes, and positive controls) for these assays are being distributed by PAHO/WHO throughout the Region.

Please find below the suggested algorithms for OPXV initial PCR or for MPXV specific initial PCR.

Molecular detection, Algorithm 1



Molecular detection, Algorithm 2



Interpretation of the results and data reporting

Confirmation of MPXV infection should consider clinical and epidemiological information.

Positive detection using an OPXV PCR assay followed by confirmation of MPXV via PCR and/or sequencing, or positive detection using MPXV PCR assay in suspected cases in endemic and nonendemic areas indicates confirmation of MPXV infection.

While it is preferable to perform MPXV specific confirmatory testing, positive detection using OPXV PCR assay is considered sufficient for laboratory confirmation of suspected cases in non-endemic countries.

Genetic sequence data (GSD) may also provide valuable information to help understand the origins, epidemiology, and characteristics of the virus, for example whether cases arise from a single introduction or multiple introductions from other locations. Countries and laboratories are encouraged to share GSD, including raw data whenever possible in a timely manner through the available public access databases.

According to the International Health Regulations (IHR), all monkeypox confirmed cases should be notified within 24 hours through official IHR channels.

Differential diagnosis

It is important to consider other potential causes of discrete skin lesions or a disseminated rash and other etiologies for similar-appearing skin lesions at the different stages of development including herpes simplex virus, varicella zoster virus, molluscum contagiosum virus, enterovirus, measles, scabies, Zika, Chikungunya, dengue, Treponema pallidum (syphilis), bacterial skin infections, medication allergies, parapoxviruses and chancroid, among others.

Sample, collection material and storage temperature for MPXV diagnostic and differential purpose

Specimen type	Collection materials*	Storage temperature	Collection purpose
Skin lesion material, including: • Swabs of lesion exudate • Lesion roofs • Lesion crusts	Dacron or polyester flocked swabs with VTM or dry swab	Refrigerate (2-8 °C) or freeze (- 20°C or lower) within 1 hour of collection; -20°C or lower after 7 days	Recommended for diagnosis
Oropharyngeal swab	Dacron or polyester flocked swabs with VTM or dry swab	Refrigerate (2-8 °C) or freeze (- 20°C or lower) within 1 hour of collection; -20°C or lower after 7 days	Recommended for diagnosis if feasible, in addition to skin lesion material
Serum	Serum-separating tubes	Refrigerate (2-8 °C) or freeze (- 20°C or lower) within 1 hour of collection; -20°C or lower after 7 days	To be considered for serology to aid diagnosis or research (following ethics guidelines)
Plasma	collection tube with EDTA	Refrigerate (2-8 °C) or freeze (- 20°C or lower) within 1 hour of collection; -20°C or lower after 7 days	To be considered for serology to aid diagnosis or research (following ethics guidelines)

*Aside from specific collection materials indicated, other requisite materials and equipment include: transport containers and specimen collection bags and triple packaging, coolers and cold packs or dry ice, sterile blood-drawing equipment (e.g. needles, syringes and tubes), labels and permanent markers, PPE, and materials for decontamination of surfaces.

References

Pan American Health Organization. Epidemiological Alert Monkeypox in non-endemic countries - 20 May 2022. Washington, DC: PAHO; 2022. Available from: https://www.paho.org/en/documents/epidemiological-alert-monkeypox-non-endemic-countries-20-may-2022

World Health Organization. Laboratory testing for the monkeypox virus. Geneva: WHO; 2022. Available from: https://www.who.int/publications/i/item/WHO-MPX-laboratory-2022.1

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Li Y, Zhao H, Wilkins K, Hughes C, Damon IK. Real-time PCR assays for the specific detection of monkeypox virus West African and Congo Basin strain DNA. J Virol Methods. 2010 Oct;169(1):223-7. doi: 10.1016/j.jviromet.2010.07.012

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Annex 1

Monkeypox virus (MPVX)
Real-time PCR protocol

Assays for the generic detection of MPXV (species: *Monkeypox virus*, genus: *Orthopoxvirus*) and the detection of its two clades:[1]

- Assay with G2R_G primers and probe: detects all MPXV strains
- Assay with G2R_WA primers and probe: detects Western African clade viruses
- Assay with C3L primers and probe: detects Congo Basin clade viruses
- Primers and probes sequences at the end of the document.
- All probes are hydrolysis ("TaqMan") probes labelled with the FAM dye and the BHQ-1 quencher.

1. Master mixes

Master mixes for each assay (G2R_G, G2R_WA or C3L) should be prepared separately.

Component	Volume per reaction **EXPRESS qPCR Supermix Universal** [2]	Volume per reaction **TaqMan® Universal PCR Master Mix** [3]
water (RNAse/DNAse free) [4]	3.0 µl	3.0 µl
reaction buffer (2x) [4]	10.0 µl	10.0 µl
forward primer (10 µM)	0.8 µl	0.8 µl
reverse primer (10 µM)	0.8 µl	0.8 µl
probe (10 µM)	0.4 µl	0.4 µl
Total per reaction	**15 µl**	

2. DNA

Add **5 µl** of DNA to the 15 µl of master mix (total reaction volume: 20 µl)

Include positive and negative **controls** to assess the validity of the run.

[1] Li *et al.*, *Journal of Virological Methods* **169**, 223–7 (2010).

[2] Invitrogen, cat. no.: 11785-200, 11785-01K, 11795-200 or 11795-01K.

[3] Applied Biosystems, cat. no.: 4304437, 4364338, 4364340, 4305719, 4318157 or 4326708.

[4] The volumes are for the indicated kits and should be adjusted when other kits are used.

3. Cycling[5]

G2R_G assay or C3L assay	
EXPRESS qPCR Supermix Universal	**TaqMan® Universal PCR Master Mix**
2 steps:	2 pasos de:
50°C - 2 minutes (UNG incubation)	50°C - 2 minutes (UNG incubation)
95°C - 6 minutes (polymerase activation)	95°C - 10 minutes (polymerase activation)
45 PCR cycles:	45 PCR cycles:
95°C - 15 seconds	95°C - 15 seconds
60°C - 30 seconds **(acquire fluorescence at this step)**	60°C - 30 seconds **(acquire fluorescence at this step)**

G2_WA assay	
EXPRESS qPCR Supermix Universal	**TaqMan® Universal PCR Master Mix**
2 pasos de:	2 pasos de:
50°C - 2 minutes (UNG incubation)	50°C - 2 minutes (UNG incubation)
95°C - 6 minutes (polymerase activation)	95°C - 10 minutes (polymerase activation)
45 PCR cycles:	45 PCR cycles:
95°C - 15 seconds	95°C - 15 seconds
62°C - 30 seconds **(acquire fluorescence at this step)**	62°C - 30 seconds **(acquire fluorescence at this step)**

[5] The duration of UNG incubation and polymerase activation are for the indicated kits and should be adjusted when other kits are used.

4. Primers and probes sequences

G2R_G assay (MPXV generic detection)	
G2R_G forward primer	5'-GGAAAATGTAAAGACAACGAATACAG
G2R_G reverse primer	5'-GCTATCACATAATCTGGAAGCGTA
G2R_G probe	5'FAM-AAGCCGTAATCTATGTTGTCTATCGTGTCC-3'BHQ1

G2_WA assay (detection of Western African clade viruses)	
G2R_WA forward primer	5'-CACACCGTCTCTTCCACAGA
G2R_WA reverse primer	5'-GATACAGGTTAATTTCCACATCG
G2R_WA probe	5'FAM-AACCCGTCGTAACCAGCAATACATTT-3'BHQ1

C3L assay (detection of Congo Basin clade viruses)	
C3L forward primer	5'-TGTCTACCTGGATACAGAAAGCAA
C3L reverse primer	5'-GGCATCTCCGTTTAATACATTGAT
C3L probe	5'FAM-CCCATATATGCTAAATGTACCGGTACCGGA-3'BHQ1